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The polyribosomal protein bound to the 3′ end of histone mRNA can function in histone pre-mRNA processing

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ABSTRACT

Cell cycle-regulated histone mRNAs end in a conserved 26-nt sequence that can form a stem-loop with a six-base stem and a four-base loop. The 3′ end of histone mRNA has distinct functions in the nucleus and in the cytoplasm. In the nucleus it functions in pre-mRNA processing and transport, whereas in the cytoplasm it functions in translation and regulation of histone mRNA stability. The stem-loop binding protein (SLBP), present in both nuclei and polyribosomes, is likely the *trans*-acting factor that binds to the 3′ end of mature histone mRNA and mediates its function. A nuclear extract that efficiently processes histone pre-mRNA was prepared from mouse myeloma cells. The factor(s) that bind to the 3′ end of histone mRNA can be depleted from this extract using a biotinylated oligonucleotide containing the conserved stem-loop sequence. Using this depleted extract which is deficient in histone pre-mRNA processing, we show that SLBP found in polyribosomes can restore processing, suggesting that SLBP associates with histone pre-mRNA in the nucleus, participates in processing, and then accompanies the mature mRNA to the cytoplasm.

Keywords: cell cycle regulation; histone mRNA; RNA processing; RNA-binding protein; stem-loop binding protein

INTRODUCTION

Cell cycle-regulated histone mRNAs are the only metazoan mRNAs that do not end in a polyA tail. Instead they end in a conserved 26-nt sequence that can form a stem-loop, with a six-base stem and four-base loop (Marzluff, 1992). The 3′ end of histone mRNA is the *cis*-acting element responsible for about 70% of cell cycle regulation of histone mRNAs, participating in the regulation of both pre-mRNA processing and histone mRNA stability (Stauber et al., 1986; Stauber & Schümperli, 1988; Harris et al., 1991). Identical 3′ ends are present on all cell cycle-regulated histone mRNAs, a total of about 70 mRNAs in mammals. Therefore, the cell can identify a histone mRNA by its 3′ end and use this element to direct the coordinate regulation of all replication-dependent histone mRNAs.

The 3′ untranslated region and the polyA tail at the 3′ end of most mRNAs participate in a variety of functions in mRNA metabolism. These include a role in translation (McGrew & Richter, 1990; Munroe & Jacobson, 1990; Gallie, 1991; Gebauer et al., 1994) and in determining the half-life of the mRNA (Sachs, 1993). Two polyA-binding proteins associate with the polyA tail. PABII, a 50-kDa polypeptide, associates with the emerging polyA tail and increases the processivity of polyA polymerase (PAP) from 5 nt/min to 1,500 nt/min (Wahle, 1991; Keller, 1995). PAB, a 70-kDa polypeptide, binds to the polyA tail on cytoplasmic mRNAs (Adam et al., 1986; Sachs et al., 1987) and is not present in the nucleus (Görlach et al., 1994). The functions of the polyA tail on cytoplasmic mRNAs are mediated through its interactions with PAB (Sachs et al., 1987; Bernstein & Ross, 1989; Sachs & Davis, 1989; Jackson & Standart, 1990). At some step between the completion of polyadenylation and transport to the cytoplasm, the polyA binding proteins are exchanged.

The 3′ end of cell cycle-dependent histone mRNAs probably fulfills the function of the polyA tail on other mRNAs (Marzluff, 1992). We have previously shown a role for the 3′ end of histone mRNAs in the regulation of histone mRNA degradation (Pandey & Marzluff, 1987) and in translation (Sun et al., 1992). Formation

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of histone mRNAs requires a single RNA processing reaction, cleavage of the pre-mRNA just after the stem-loop (Gick et al., 1986). Cleavage requires both the highly conserved stem-loop and a loosely conserved purine-rich sequence that interacts with U7 snRNP (Mowry & Steitz, 1987b; Cotten et al., 1988; Soldati & Schümperli, 1988). *Trans*-acting factors involved in this histone pre-mRNA processing include a factor that interacts with the stem-loop, termed the hairpin binding factor (HBF) (Mowry et al., 1989; Vasserot et al., 1989; Melin et al., 1992), the U7 snRNP, and a poorly characterized heat-labile factor (Gick et al., 1987).

Polyribosome-associated histone mRNAs contain a 45-kDa protein, the stem-loop binding protein (SLBP), bound to the 3′ end of the mRNA (Pandey et al., 1991), with a single SLBP molecule bound per histone mRNA (Hanson et al., 1996). SLBP binds specifically to the 3′ end of histone mRNA, and binding requires not only the specific stem-loop sequence, but also the conserved flanking nucleotides (Williams & Marzluff, 1995). SLBP, which is assayed by a mobility shift assay and UV-crosslinking, is also present in the nucleus (Pandey et al., 1991). Here we test the hypothesis that SLBP and the nuclear pre-mRNA processing factor, HBF, are related. We show that SLBP from either the nucleus or the polyribosomes can substitute for HBF in the histone pre-mRNA processing reaction.

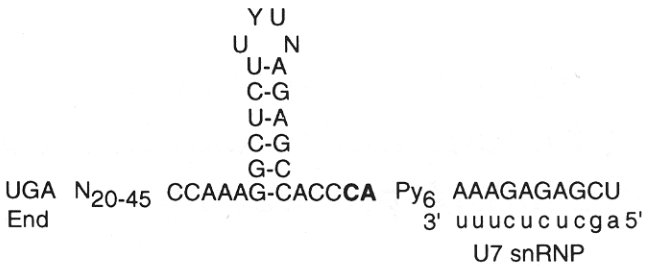


FIGURE 1. 3′ end of histone pre-mRNA. The consensus 3′ end of vertebrate histone pre-mRNAs is shown. There is a conserved 26-nt sequence that includes the stem-loop. Distance between the stem-loop and the U7 snRNP-binding site is variable. U7 snRNA-binding site is loosely conserved in vertebrates. Base pairing between the U7 snRNA and the histone pre-mRNA is shown. Mature histone mRNA is produced by cleavage at the CA (shown in bold), five bases after the stem-loop.

RESULTS

The consensus sequence for the 3′ end of replication-dependent histone pre-mRNAs with the conserved 26 nt present in all metazoan histone mRNAs is shown in Figure 1. The U7 snRNP binding site is indicated. The histone pre-mRNA is cleaved five bases from the end of the stem after the CA (Scharl & Steitz, 1994). In an in vitro system, cleavage occurs in the absence of nucleotides and divalent ions (Gick et al., 1986; Mowry & Steitz, 1987b). A synthetic 320-nt histone pre-mRNA that contains the 3′ end of the histone H2a-614 coding region, the 3′ untranslated region, the processing signal, and an additional 54 nt of 3′ flanking sequence was synthesized and incubated in an extract prepared from mouse myeloma cell nuclei. Cleavage of the synthetic pre-mRNA occurred efficiently, with up to 50% of the substrate being cleaved in 30–40 min (Fig. 2A).

Two *trans*-acting factors, the U7 snRNP and HBF, interact with the two conserved sequence elements (Mowry & Steitz, 1987b; Cotten et al., 1988; Mowry et al., 1989; Vasserot et al., 1989; Melin et al., 1992). Factors that interact with the stem-loop can be detected by two different assays: a functional assay for histone pre-mRNA processing, and a binding assay for specific binding to the stem-loop. It is not clear whether the same factors are detected by both assays. We refer to HBF as the factor required for processing, defined by the functional assay. We refer to SLBP as the factor that binds to the stem-loop detected by the mobility-shift assay. SLBP and HBF could be totally different, could be the same factor, or SLBP could be a component of HBF.

There are differing reports on the requirement of HBF and the stem-loop sequence for in vitro processing, depending on the substrate and the cell line used to prepare the extract (Streit et al., 1993). However, there is agreement that the stem-loop sequence is not absolutely essential for processing (Mowry et al., 1989; Vasserot et al., 1989). Thus, incubation of the extract with increasing amounts of a 30-nt RNA competitor that contains the stem-loop sequence only inhibited processing 90–95%, but did not completely abolish it (Fig. 2B, lanes 2–5). The residual processing probably represents processing due to the U7 snRNP and other *trans*-acting factors (e.g., the heat-labile factor [Gick et al., 1987]) functioning in the absence of the HBF. Two mutant oligonucleotides that did not bind SLBP well, SL(RS) with the stem reversed, and SL(2′OCH_3) containing the wild-type sequence with 2′OCH_3 ribonucleotides at each position, had no effect on processing (Fig. 2C, lanes 4, 5). As reported previously by others (Mowry & Steitz, 1987b; Cotten et al., 1988), processing was absolutely dependent on U7 snRNP, because incubation with a 2′OCH_3 oligoribonucleotide complementary to the 5′ end of U7 snRNA completely abolished processing (Fig. 2C, lane 3) by binding the 5′ end of U7 snRNA (Smith et al., 1991).

A depleted extract that can be used to functionally assay the hairpin factor

In order to develop a functional assay for HBF, we developed a method to selectively deplete HBF from the nuclear extract. A biotinylated RNA, $(WT)_{biotin}$, containing the stem-loop sequence with biotin at the 3′ end, was synthesized. The $(WT)_{biotin}$ RNA had two 2′OCH_3 ribonucleotides incorporated at the 5′ and 3′ end of the oligonucleotide to protect against exonucle-

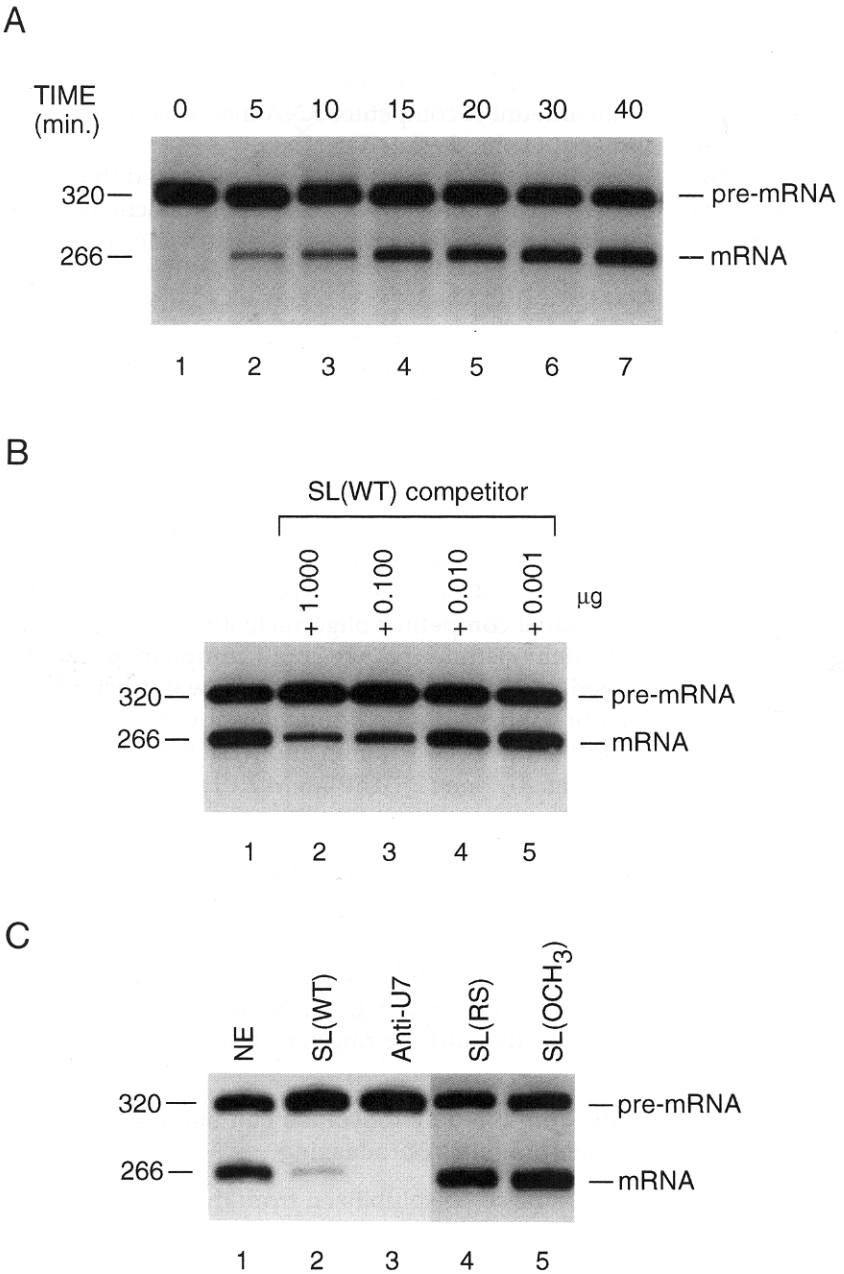


FIGURE 2. Processing of histone pre-mRNA in vitro. **A:** Radiolabeled 320-nt synthetic histone pre-mRNA was incubated with a nuclear extract from mouse myeloma cells as described in the Materials and methods. At the indicated times, the RNAs were isolated and resolved by electrophoresis on an 8% polyacrylamide–7-M urea gel. RNAs were detected by autoradiography. **B:** 320-nt pre-mRNA was incubated with a nuclear extract from mouse myeloma cells for 30 min in the presence of varying amounts of a 30-nt wild-type stem-loop oligonucleotide indicated above each lane. Histone pre-mRNA processing was detected as in A. **C:** 320-nt pre-mRNA was incubated with a nuclear extract in the absence (lane 1) or the presence of 1 μg of the following oligonucleotides: lane 2, SL(WT) competitor; lane 3, antisense U7 2′(OCH_3) oligoribonucleotide; lane 4, SL(RS) competitor SL(RS) RNA with the six base pairs in the stem reversed; lane 5, SL(OCH_3) competitor stem-loop RNA containing all 2′OCH_3 nucleotides.

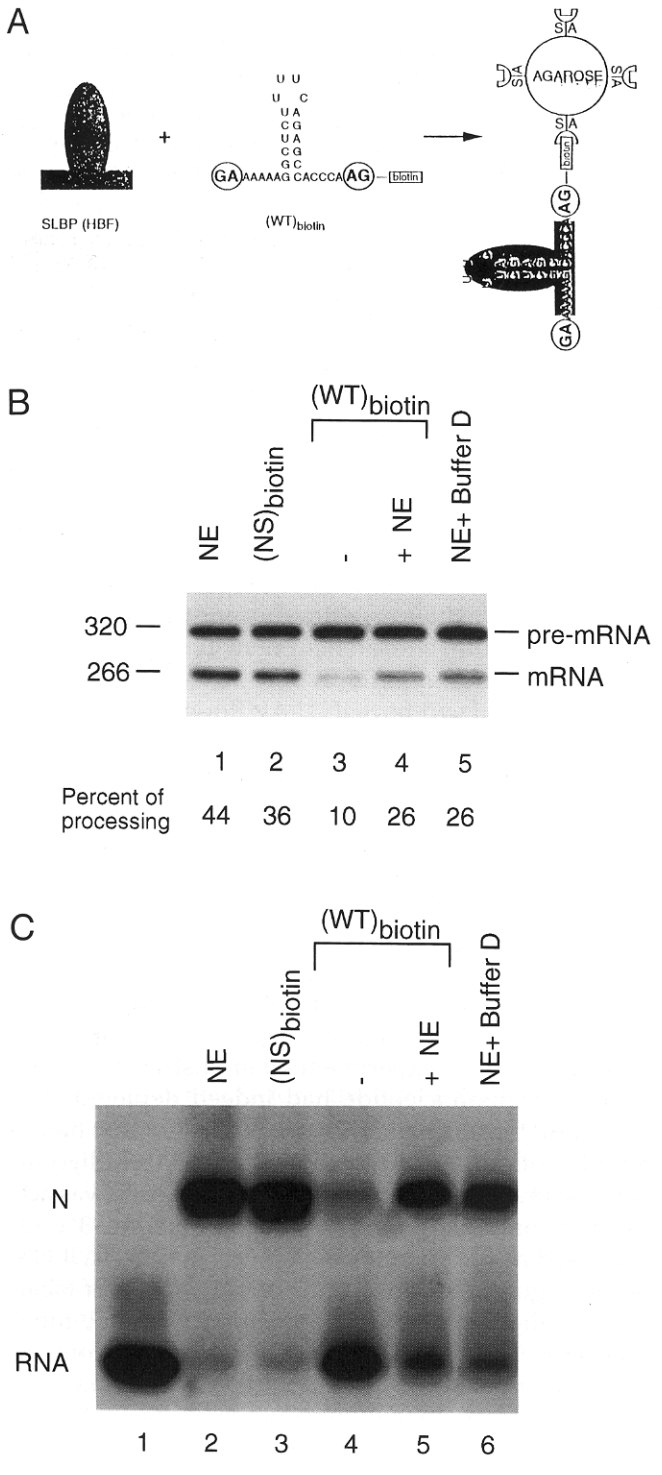
olytic cleavage. Because HBF and SLBP both interact with the stem-loop, incubation of the $(WT)_{biotin}$ RNA with the extract should bind both HBF and SLBP. After incubation with the extract, the biotinylated RNA and any associated factors were removed by addition of streptavidin agarose (Fig. 3A). A control extract was incubated in parallel with a non-specific oligonucleotide $(NS)_{biotin}$, which does not bind to HBF or SLBP. The sequence of the $(NS)_{biotin}$ oligonucleotide is given in the Materials and methods. Incubation with the $(NS)_{biotin}$ oligonucleotide resulted in only a slight reduction in processing (Fig. 3B, lane 2). After depletion with the $(WT)_{biotin}$ oligonucleotide, processing was reduced by about 80% (Fig. 3B, lane 3), indicating that HBF was depleted. There is a similar level of residual processing activity in the depleted extract as in an extract with the stem-loop competitor added (compare Fig. 2C, lane 2 with Fig. 3B, lane 3).

Several control experiments demonstrated that the $(WT)_{biotin}$ oligonucleotide had indeed depleted HBF and SLBP from the extract. One possibility that had to be ruled out was that there was residual free oligonucleotide present in the depleted extract which was acting as a competitor. Because the biotin (at the 3′ end) was used as the primer for chemical synthesis, 100% of the oligonucleotides were biotinylated. After labeling the oligonucleotide at the 5′ end with ^{32}P, more than 95% of the oligonucleotide was removed from the extract by the streptavidin agarose (data not shown). To test for the presence of residual competitor RNA re-

maining in the extract, the depleted extract was mixed with an undepleted extract. If there was residual competitor RNA in the depleted extract, then processing of histone pre-mRNA would be inhibited in the undepleted extract. Equal amounts of undepleted nuclear extract were mixed with the depleted extract (Fig. 3B, lane 4) or with buffer D (Fig. 3B, lane 5) and the processing activity tested. Because the nuclear extract was diluted, there was reduced processing activity compared with the standard reaction (Fig. 3B, compare lanes 1 and 5). However, addition of the depleted extract had no effect on the processing activity (Fig. 3B, lane 4). This demonstrates that there was not a significant amount of competitor RNA that could inhibit HBF present in the depleted extract.

Mobility shift experiments demonstrated that there was also very little (<10%) residual SLBP activity in the depleted extract (Fig. 3C, lane 4). The same analysis of SLBP activity was done to determine whether competitor RNA was present in the depleted extract. Addition of the undepleted nuclear extract to the depleted extract did not significantly reduce SLBP activity (Fig. 3C, lane 5) compared with dilution of the undepleted extract (Fig. 3C, lane 6). This demonstrates that there was not sufficient oligonucleotide remaining in the depleted extract to act as a competitor for SLBP binding. The SLBP competition assay is more sensitive than the processing assay, and the slight reduction in binding observed (Fig. 3C, lane 5) may be due to small amounts of residual competitor oligonucleotide.

To demonstrate that we could complement the depleted extract, the nuclear extract was fractionated by phosphocellulose chromatography. SLBP activity bound tightly to the column and was eluted with 0.6 M KCl (Fig. 4A, lane 2). The 0.6 M KCl fraction does not contain any processing activity, presumably because it lacks U7 snRNP. Addition of the 0.6 M KCl fraction to the depleted extract restored pre-mRNA processing (Fig. 4B, lane 4), indicating that the 0.6 M KCl fraction also contains HBF. Thus, the HBF (and SLBP) can be selectively depleted from the nuclear extract and then processing restored by the addition of a fraction that contains HBF and the nuclear SLBP.



SLBP from the polyribosomes can support histone pre-mRNA processing

SLBP can also be solubilized from the polyribosomes by extraction with 0.8 M KCl (Pandey et al., 1991).

FIGURE 3. Depletion of the factors that interact with the stem-loop from a nuclear extract. **A:** Strategy used to deplete the extract of HBF using the $(WT)_{biotin}$ RNA is shown. Circled nucleotides indicate $2'OCH_3$ nucleotides that were included to increase stability of the RNA. **B:** Nuclear extract from mouse myeloma cells was incubated with $(NS)_{biotin}$ oligoribonucleotide (lane 2) or $(WT)_{biotin}$ oligoribonucleotide (lane 3) as described in the Materials and methods. Extracts were then incubated with the synthetic 320-nt histone pre-mRNA for 30 min at 32 °C and RNAs resolved by electrophoresis, detected by autoradiography, and quantified using a PhosphorImager. Lane 4, 5 μL of undepleted extract was mixed with 12 μL of the depleted extract; lane 5, 5 μL of undepleted extract was diluted with 12 μL of buffer D; lane 1, 17 μL of undepleted nuclear extract. **C:** The same extracts as in B were assayed for SLBP activity using a mobility shift assay. A radiolabeled 30-nt RNA probe containing the stem-loop was incubated with the extract and the complexes then detected by gel electrophoresis under native conditions. Lane 1, no extract; lane 2, undepleted extract; lane 3, depleted with $(NS)_{biotin}$ oligoribonucleotide; lane 4, depleted with $(WT)_{biotin}$ oligoribonucleotide; lane 5, 5 μL of undepleted extract was mixed with 12 μL of depleted extract; lane 6, 5 μL of undepleted extract was diluted with 12 μL of buffer D.

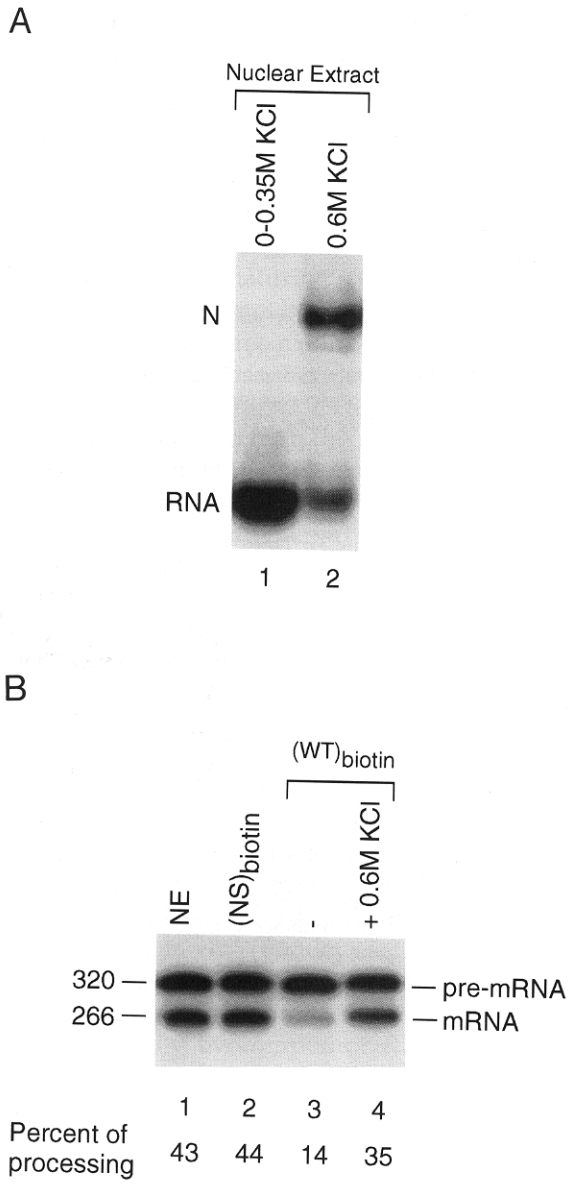


FIGURE 4. Restoration of processing by a nuclear factor. **A:** Fractions from the phosphocellulose column were assayed for SLBP activity using the mobility shift assay as in Figure 3C. Lane 1, probe was incubated with the combined fractions eluting from 0.1 to 0.35 M KCl. Lane 2, probe was incubated with the fraction eluting at 0.6 M KCl. **B:** 320-nt histone pre-mRNA was incubated with different extracts for 30 min and histone pre-mRNA processing assayed as in Figure 2A. Extracts used were: lane 1, a nuclear extract; lane 2, the same extract depleted using $(NS)_{biotin}$ RNA; or lane 3, $(WT)_{biotin}$ RNA. Depleted nuclear extract used in lane 3 was supplemented with the 0.6 M KCl fraction of a nuclear extract from the phosphocellulose column (lane 4).

Addition of increasing amounts of a polyribosomal extract to the depleted extract restored processing in a concentration-dependent manner (Fig. 5A, lanes 4, 5). Polyribosomal SLBP restored processing to the same extent as the nuclear 0.6 M KCl fraction, consistent with the possibility that SLBP is the same as or a component of HBF. The polyribosomal extract did not contain any processing or nuclease activity (Fig. 5A, lanes 6, 7) when assayed under these conditions. The exonuclease activity present in these extracts, which can degrade histone mRNA, is absolutely dependent on divalent ions for activity and is not active under these conditions (Ross et al., 1987; Caruccio & Ross, 1994).

A second method was used to demonstrate reconstitution of histone pre-mRNA processing. The nuclear extract was fractionated by phosphocellulose chromatography. The fractions eluting through the 0.35 M KCl step were combined and concentrated by ammonium sulfate precipitation. These fractions did not support processing (Fig. 5B, lanes 2) and did not contain any SLBP activity (Fig. 4A, lane 1). SLBP was recovered from the column by elution with 0.6 M KCl. Addition of either the 0.6 M KCl fraction from the nuclear extract (Fig. 5B, lane 3) or the 0.6 M KCl fraction from the polyribosomal extract (Fig. 5B, lane 4) restored processing equally well. The level of processing observed in these reconstituted extracts was lower than that observed with nuclear extract (Fig. 5B, lane 1), presumably because of the more extensive handling of these extracts. These results demonstrate that SLBP solubilized from the polyribosomes was as effective in supporting processing as the nuclear activity, making it likely that the polyribosomal SLBP either is HBF or is an integral component of HBF.

DISCUSSION

Many proteins participate in pre-mRNA processing in the nucleus and then dissociate from the mRNA prior to its transport to the cytoplasm. These include a cap-binding protein (Izaurralde et al., 1992), the polyA-binding protein 2 (PABII) (Wahle, 1991), and several of the hnRNP proteins (Piñol-Roma & Dreyfuss, 1992). Other proteins, notably the hnRNP A proteins, may associate with the pre-mRNA and then accompany the mRNA to the cytoplasm after processing (Piñol-Roma & Dreyfuss, 1992). These proteins then return to the nucleus after being removed from the mRNA, possibly during translation. There is also evidence that *rev* shuttles between the nucleus and cytoplasm, in its role in facilitating transport of unspliced HIV RNA (Kalland et al., 1994). Additionally, there are distinct cap-binding proteins (Rhoads, 1988) and polyA-binding proteins (Adam et al., 1986; Görlach et al., 1994) present in the cytoplasm associated with polyribosomal mRNA. The eucaryotic initiation factor 4E (eIF-4e) binds to the cap and assists in translation initiation (Rhoads, 1988). Thus, the two proteins that bind specific structures on either end of polyadenylated mRNAs have distinct counterparts in the nucleus and the cytoplasm.

In contrast, our results with histone mRNA suggest that the same polypeptide interacts with the stem-loop in histone pre-mRNA in the nucleus and subsequently translocates with the mature mRNA to the cytoplasm. We have previously shown that similar factors that recognize the stem-loop at the 3′ end of histone mRNA are present in both the nucleus and the polyribosomes

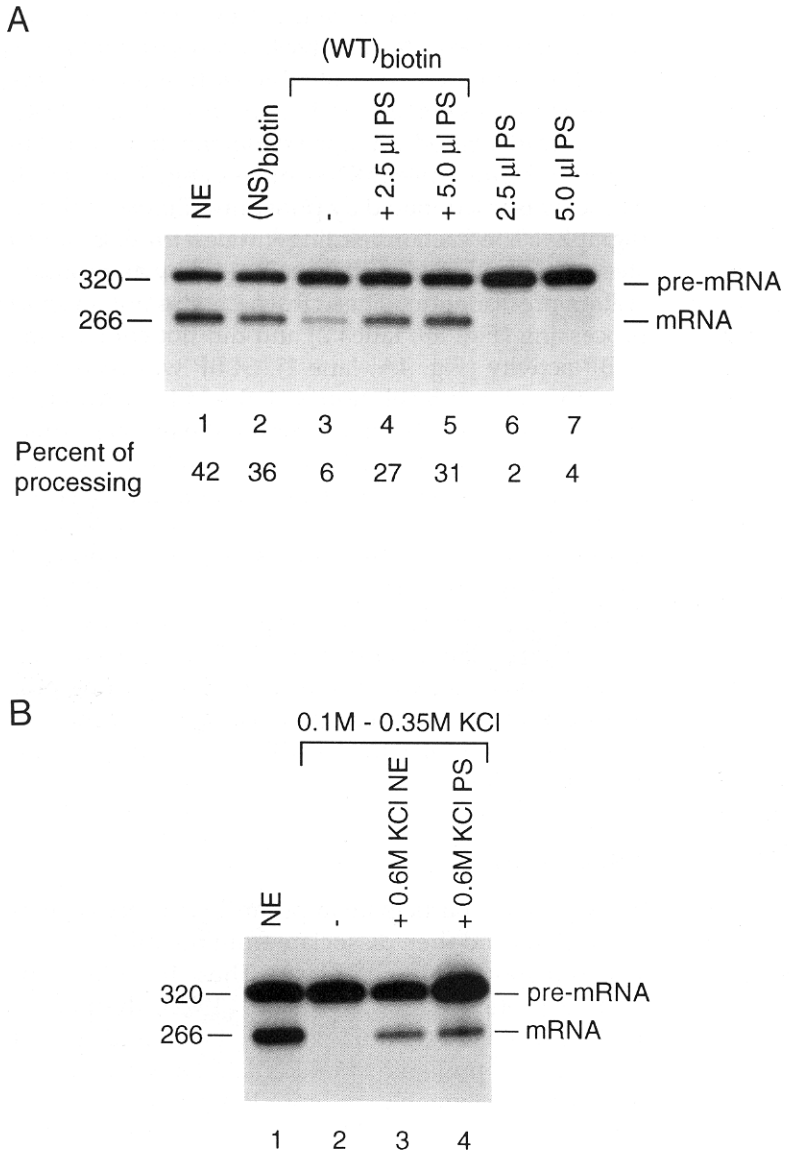


FIGURE 5. Polyribosomal SLBP can restore processing. **A:** Nuclear extract was incubated with $(NS)_{biotin}$ oligoribonucleotide (lane 2) or $(WT)_{biotin}$ oligoribonucleotide (lane 3) as in Figure 3, and histone pre-mRNA processing assayed. Lane 1 is processing in the intact nuclear extract. Increasing amounts of a 0.8 M KCl extract of polyribosomes was added to the depleted extract from lane 3 in lanes 4 and 5. In lanes 6 and 7, the same amount of polyribosomal extract was incubated with the pre-mRNA under processing conditions to demonstrate there was no nuclease activity in the polyribosomal extract. Histone pre-mRNA processing was assayed as in Figure 2 and quantified on a Phosphorimager. **B:** Nuclear extract was applied to phosphocellulose in 0.1 M KCl and the fractions eluting from 0.1 to 0.35 M KCl were combined, concentrated, and dialyzed. This fraction contained U7 snRNP and had no processing activity (lane 2). A polyribosomal extract was also chromatographed on phosphocellulose and the 0.6 M KCl fraction containing SLBP recovered. Equal amounts of SLBP from the 0.6 M KCl fraction of the phosphocellulose column from either a nuclear extract (lane 3) or a polyribosomal extract (lane 4) were added to the combined 0.1–0.35 M fractions.

(Pandey et al., 1991). There is a 45-kDa polypeptide, SLBP, that directly contacts the stem-loop as determined by UV crosslinking (Pandey et al., 1991). Several other groups have defined a histone pre-mRNA processing factor, HBF, that interacts with the stem-loop and greatly stimulates histone pre-mRNA processing (Mowry et al., 1989; Vasserot et al., 1989; Melin et al., 1992). HBF has not been well-characterized, although it has been reported that it is recognized by anti-Sm antibodies (Mowry & Steitz, 1987a; Vasserot et al., 1989). SLBP is present as a monomer bound to the 3′ end of polyribosomal histone mRNA (Hanson et al., 1996). SLBP does not react with Sm-antibodies, as judged by the failure of a polyclonal antibody to deplete SLBP or to alter the mobility shift (A.S. Williams & W.F. Marzluff, unpubl.). The processing factor, HBF, could be the same as SLBP. However, HBF need only be present in catalytic amounts and hence could be of very low abundance and not detected in the mobility shift assay. Our results are consistent with the possibility that SLBP found in the polyribosomes is the same as the HBF required for pre-mRNA processing. Alternatively, SLBP, present in the polyribosomes as a monomer, may be one of the components of the HBF. The molecular size of HBF is not known, but SLBP could be one component of a larger complex required for processing. If this is the case, then the depleted extract must contain sufficient amounts of the other components of the HBF to assemble HBF after addition of SLBP.

The SLBP has been identified as a 45-kDa polypeptide by UV-crosslinking (Pandey et al., 1991). The nuclear and polyribosomal SLBP polypeptides are identical as judged by binding specificity (Williams & Marzluff, 1995) and partial proteolysis after UV-crosslinking (Hanson et al., 1996). SLBP binds very tightly

to its target RNA, the 30 nt at the 3′ end of histone mRNA, and does not dissociate in vitro over several hours (Williams & Marzluff, 1995). Like several other sequence-specific RNA-binding proteins (e.g., PAB [Sachs & Kornberg, 1990], a cytoplasmic polyadenylation determinant, CPEB [Hake & Richter, 1994], and U2aF [Zamore et al., 1992]), the SLBP can only be recovered from affinity resins containing the stem-loop by treatment with denaturing solvents (Hanson et al., 1996). Thus, we have not been able to reconstitute the processing activity by recovering the bound SLBP from the streptavidin agarose. Because the SLBP is present at relatively low concentrations (probably one molecule per histone mRNA) in both the nucleus and the polyribosomes, and both the polyribosomal and nuclear extracts are a complex mixture of polypeptides, it has not been possible to identify a particular protein band as the SLBP in the phosphocellulose fractions used in the complementation experiments. Complete purification of the SLBP is currently in progress.

The results we report here suggest the following model for histone mRNA formation. One of the factors required for histone pre-mRNA processing, HBF, associates with the pre-mRNA. After cleavage of the pre-mRNA, at least one of the components of HBF, SLBP, remains with the mature mRNA and accompanies it to the cytoplasm. Our previous studies of the properties of SLBP support this model. Mutations in the stem-loop have a parallel effect on SLBP binding and on histone processing in vivo (Pandey et al., 1994), consistent with the model that SLBP plays a role in processing. SLBP binds very tightly to the stem-loop and is not displaced readily by competitor RNAs (Williams & Marzluff, 1995), consistent with the possibility that it remains bound for the lifetime of the histone mRNA. Previously, we have shown that a histone mRNA with a mutation in the stem-loop that reduces the affinity for SLBP is not efficiently transported, supporting the idea that SLBP is directly involved in this process (Williams et al., 1994). In addition, because the histone mRNA 3′ end is required for translation (Sun et al., 1992) and regulation of histone mRNA degradation (Pandey & Marzluff, 1987), SLBP is likely to play a role in both of these processes.

MATERIALS AND METHODS

Production of synthetic pre-mRNAs and competitor RNAs

The histone pre-mRNA was derived from the mouse histone H2a-614 gene (Hurt et al., 1989). The pre-mRNA extended from the *Pst* I site at codon 85 to the *Hpa* II site 35-nt past the U7 binding site and has been used previously (Pandey et al., 1994). A 320-nt synthetic histone pre-mRNA containing the H2a-614 3′ end plus 19 nt of polylinker was synthesized from a plasmid template linearized at the *Hin*d III site using T7 RNA polymerase. The pre-mRNA was capped by the addition of $^{7}CH_3GpppG$ to prime the synthesis. The processed RNA from this pre-mRNA is 266 nt. Competitor RNAs (30 nt) were synthesized from oligonucleotide templates (Milligan et al., 1987) as described previously (Pandey et al., 1991). The 2′OCH_3 stem-loop competitor was synthesized on an oligonucleotide synthesizer. Biotinylated RNA was synthesized on an Applied Biosystems Synthesizer with the biotin attached to the CPG matrix and a 15-atom linker between the biotin and the last nucleotide (BiotinTEG-CPG, Glen Research). The RNAs were synthesized with 2′OCH_3 ribonucleotides incorporated at the 5′ and 3′ end of the RNA sequence to protect the RNA from nucleases (see Fig. 3A). A synthetic biotinylated 2′OCH_3 RNA complementary to the 5′ end of U7 snRNA, the same oligonucleotide used by Smith et al. (1991), was used to inhibit U7 snRNP.

Preparation of mouse myeloma nuclear extract

Mouse myeloma cells were cultured in suspension in Dulbecco's Modified Eagle Medium plus 10% horse serum and harvested at a concentration of 4–6 × 10^5 cells/mL. Nuclei were isolated essentially by the method of Shapiro et al. (1988) with minor modifications. All procedures were conducted at 4 °C. Cells were harvested by centrifugation (800 × *g*) and washed once with ice-cold phosphate-buffered saline. The packed cell volume was measured and four volumes of buffer A (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol [DTT], 0.75 mM spermidine, 0.15 mM spermine) was added. The cells were swollen for 3–8 min and broken by homogenization in a Dounce homogenizer (tight pestle) until more than 90% of the cells were lysed. One-tenth volume of restore buffer (67% sucrose, 50 mM HEPES, pH 7.9, 0.75 mM spermidine, 0.15 mM spermine, 10 mM KCl, 0.2 mM EDTA, and 0.1 mM DTT) was added and the nuclei recovered by centrifugation at 800 × *g* for 2 min. The supernatant was removed (cytosolic fraction) and nuclei were resuspended in low-salt buffer (20 mM HEPES, pH 7.9, 25% glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, and 20 mM NaCl). High-salt buffer (20 mM HEPES, pH 7.9, 25% glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, and 1.2 M NaCl) was added dropwise with swirling until the first evidence of any nuclear lysis (final NaCl concentration approximately 0.23 M). The nuclear suspension was rocked gently for 1 h at 4 °C. The nuclei were removed by centrifugation at 10,000 × *g* for 30 min, and the supernatant was dialyzed against buffer D (20 mM HEPES, pH 7.9, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, and 0.5 mM DTT). The extract was then centrifuged at 10,000 × *g* for 20 min to remove precipitated material, and the supernatant was stored at −80 °C in small aliquots. Typical protein concentrations were 4–6 mg/mL. We found that not allowing the nuclei to lyse during the extraction resulted in an extract with better processing efficiency then extracts prepared with higher salt (0.35–0.6 M), which resulted in lysis of the nuclei.

Preparation of polyribosomal extract from mouse myeloma cells

Purified polyribosomes were prepared exactly as described previously (Pandey et al., 1991). The resulting pellet was rinsed with buffer D and resuspended in a minimal volume

of buffer D (1 mL/3 L cells). The final salt concentration was increased to 0.8 M KCl by addition of 4 M KCl and the polyribosomes were extracted for 30 min at 4 °C by continuous rocking. The extract was centrifuged at 100,000 × *g* for 1.4 h in the TL-30 tabletop ultracentrifuge (Beckman Instruments) and the supernatant was dialyzed against buffer D. The precipitate was removed by centrifugation and the supernatant was frozen and stored at −80 °C in small aliquots. The typical protein concentration was 5 mg/mL.

Fractionation of nuclear and polyribosomal extracts by P11 phosphocellulose chromatography

P11 phosphocellulose (Whatman Laboratory Division, Maidstone, England) was equilibrated according to the manufacturer's instructions and then resuspended in buffer D. Typically, 500 μL of extract (in 0.1 M KCl, approximately 5 mg protein/mL) was applied to a 312-μL column. The column was washed with three column volumes of buffer D, and the bound proteins were eluted using a step gradient (1.2 column volumes per step) of KCl (0.35 M, 0.6 M, 0.85 M, and 1 M KCl). The SLBP activity was detected using a mobility shift assay with a radiolabeled 30-nt RNA containing the stem-loop sequence as a probe. The SLBP activity eluted in the 0.6 M KCl step. The 0.6 M KCl fractions containing SLBP were combined, concentrated by ammonium sulfate precipitation, resuspended in buffer D, and then dialyzed against buffer D. The fractions from the nuclear extract not containing SLBP activity (the flow-through, the 0.1 M KCl wash, and the 0.35 M KCl step) were also combined, precipitated, resuspended, and dialyzed.

Depletion of HBF and SLBP from nuclear extracts

Streptavidin agarose was rinsed several times with buffer D and then incubated with five volumes of nuclear extract with continuous mixing at 4 °C for 3–5 h. The streptavidin agarose was then removed by centrifugation at 1,000 × *g* and rinsed with buffer D several times before use in the depletion reaction. To deplete the nuclear extract, 2 μg of the synthetic 3′ biotinylated RNA, $(WT)_{biotin}$, containing the histone stem-loop structure was incubated with 100 μL of nuclear extract (5 mg protein/mL) for 5 min at room temperature, and then at 4 °C with rocking for 2–3 h. This amount of oligonucleotide was sufficient to maximally inhibit processing. Twenty microliters of pretreated streptavidin agarose was then added to the nuclear extract containing the biotinylated RNA and incubated with continuous rocking at 4 °C for 3 h. The streptavidin agarose and bound protein were removed by centrifugation at 1,000 × *g*, and the supernatant was frozen in small aliquots. The efficiency of SLBP depletion was measured by the mobility shift assay, and the efficiency of depletion of HBF was determined by the processing reaction. Control extracts were depleted with a mutant biotinylated 2′(OCH_3) RNA, $(NS)_{biotin}$ with the sequence GUGCCUAUCAGAA-biotin, in parallel in each experiment.

Histone pre-mRNA processing

Reactions were conducted in a final volume of 20 μL containing 17 μL nuclear extract in buffer D (85 μg protein), 10,000 cpm pre-mRNA transcript (3 × 10^4 cpm/μg; 0.33 pmol), and 20 mM EDTA. Reactions were incubated at 32 °C for 30 min, and then were stopped by the addition of NaOAC and extracted with phenol. RNA was recovered by ethanol precipitation and analyzed on an 8% polyacrylamide-7-M urea gel. RNA was detected by autoradiography and quantified using a PhosphorImager (Molecular Dynamics).

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